Enhancement of bovine oocyte fertilization *in vitro* with a bovine oviductal specific glycoprotein

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The purpose of this study was to determine the effect of a partially purified bovine oviductal glycoprotein (bOGP) on fertilization rates of bovine oocytes. The effect of albumin (control protein) or bOGP at 100 µg ml⁻¹ during the 16–18 h fertilization period was evaluated in a standard IVF system using a sperm concentration between 0.5 and 0.125 × 10⁶ spermatozoa ml⁻¹. bOGP maintained a higher ($P < 0.05$) fertilization rate (62.0% versus 31.2%) at 0.125 × 10⁶ spermatozoa ml⁻¹ compared with the albumin control. The enhancement of fertilization by bOGP was blocked by the inclusion of a specific antibody to bOGP, whereas the antibody with albumin had no effect. A 2 h gamete preincubation step was subsequently included in the IVF procedure (0.125 × 10⁶ spermatozoa ml⁻¹) to determine whether the effect of bOGP was mediated through an interaction with the oocyte, the spermatozoon or both. When oocytes were preincubated with bOGP the fertilization rates were higher ($P < 0.05$) than with the albumin control (oocytes and spermatozoa exposed to albumin), whereas preincubation of spermatozoa with bOGP did not affect fertilization rates. There was no synergistic effect of preincubating oocytes and spermatozoa with bOGP. The increase in fertilization rate achieved by preincubating oocytes with bOGP was blocked with a specific antibody to bOGP. These results suggest that the increase in fertilization rates observed when bOGP is included during the 16–18 h fertilization period are primarily mediated through the interaction of bOGP with the oocyte since the same facilitatory effect was observed with a 2 h preincubation of oocytes before IVF. The ability to block these effects with a polyclonal antibody specifically generated against bOGP shows that this biological activity is due to bOGP. In summary, bOGP enhances fertilization in bovine oocytes whether it is included during preincubation or insemination and this appears to be due to a direct effect on the oocyte.

Introduction

The mammalian oviduct is the site of fertilization and the initial stages of embryonic development. A high molecular weight oviductal glycoprotein (OGP) is synthesized and secreted by the secretory cells of the oviduct. The presence of OGP is highest during the periovulatory period and declines thereafter, suggesting a potential role during *in vivo* fertilization and the initial cleavage stages (for a review see Malette *et al.*, 1995; Verhage *et al.*, 1997).

OGP has been detected in fluid collected from the oviductal lumen of several mammalian species including baboons (Verhage *et al.*, 1989), humans (Verhage *et al.*, 1988; Rapisarda *et al.*, 1993), hamsters (Araki *et al.*, 1987; Robitaille *et al.*, 1988; Malette *et al.*, 1995), mice (Kapur and Johnson, 1985), rabbits

(Oliphant *et al.*, 1984), pigs (Buhi *et al.*, 1990), sheep (Gandolfi *et al.*, 1989; Buhi *et al.*, 1991; Murray, 1992) and cows (Boice *et al.*, 1990a; Gerena and Killian, 1990). Furthermore, OGP has been shown to associate with the zona pellucida of oocytes in hamsters (Kan *et al.*, 1990; Abe *et al.*, 1992), pigs (Buhi *et al.*, 1993), sheep (Gandolfi *et al.*, 1989) and baboons (Boice *et al.*, 1990b) in addition to the perivitelline space and blastomere membranes of embryos from hamsters, pigs, sheep and baboons (see review by Malette *et al.*, 1995; O'Day-Bowman *et al.*, 1995). In cattle, OGP has been shown to associate with the zona pellucida and perivitelline space of oocytes and the plasma membrane of oviductal embryos (Wegner and Killian, 1991; Boice *et al.*, 1992). Oviductal glycoprotein has also been shown to associate with bovine (King and Killian, 1994) and hamster (Boatman and Magnoni, 1995) spermatozoa during incubation *in vitro*, which may enhance bovine sperm capacitation (King *et al.*, 1994) and maintain bovine sperm motility and viability (Abe *et al.*, 1995). Therefore, several studies have

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Received 13 October 1997.

reported an association of OGP with both gametes. However, the effect of OGP on the fertilization process is unclear.

In bovine IVF systems sperm concentrations are routinely adjusted to maximize fertilization rates and oocytes are often exposed to high concentrations of spermatozoa. The production of oxygen radicals from high concentrations of spermatozoa (Aitken *et al.*, 1996) may adversely affect embryo development and implantation. In addition, during fertilization within the oviduct, oocytes are only exposed to a few spermatozoa (Thibault, 1973).

The present study was conducted to determine the effect of a partially purified bovine OGP (bOGP) on the fertilization rates of bovine oocytes during IVF. After preliminary studies, sperm concentrations were lowered to provide an experimental system more analogous to physiological conditions *in vivo*.

Materials and Methods

In vitro maturation and fertilization

The procedures for *in vitro* maturation and IVF were as described by Thibodeaux *et al.* (1995). Cumulus-oocyte complexes harvested from ovaries collected at an abattoir were matured for 22–24 h while in transit to the laboratory in a portable incubator (Mini-Tube of America, Madison, WI). The maturation medium was Hepes buffered TCM-199 (Sigma Chemical Co., St Louis, MO) supplemented with 10% (v/v) fetal bovine serum (Hyclone Laboratories, Logan UT), hormones (0.01 iu ml⁻¹ each of purified bovine FSH and LH; NOBL Laboratories, Sioux Center, IA), and antibiotics (100 iu penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹; Sigma Chemical Co., St Louis, MO).

After the *in vitro* maturation period, oocytes were pooled and washed twice in Hepes-buffered Tyrode's-lactate (TL-Hepes) medium (Bavister, 1989). Oocytes were then washed twice in fertilization medium consisting of modified Tyrode's-lactate medium (Bavister, 1989) supplemented with 0.25 mmol sodium pyruvate l⁻¹ (Sigma Chemical Co., St Louis, MO), antibiotics (100 iu penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹), and 6 mg fatty acid free BSA ml⁻¹ (Sigma Chemical Co., St Louis, MO; Fert-Talp). Oocytes were transferred into 50 µl drops (25–30 oocytes per drop) of fertilization medium in Petri dishes (Falcon; Scientific Products, McGraw Park, IL) that were overlaid with light mineral oil (Sigma Chemical Co., St Louis, MO) previously washed with sterile saline.

Semen from two Holstein bulls was used in the experiments (Eastern Breeders, Ithaca, NY and Select Sires, Plain City, OH). For each group of oocytes, one straw of frozen-thawed semen was layered on a 90 and 45% discontinuous Percoll gradient and centrifuged at 700 g for 30 min as described by Thibodeaux *et al.* (1995). The sperm pellet was washed twice by centrifugation (200 g for 6 min) with fertilization medium and subsequently resuspended in fertilization medium to obtain a stock sperm solution containing 1.0×10^6 spermatozoa ml⁻¹. All sperm dilutions were made from the stock solution. A 50 µl aliquot of the stock sperm cell suspension was added to the 50 µl droplet containing the oocytes.

After 16–18 h of sperm-oocyte co-incubation at 39°C and 5% CO₂ in humidified air, zygotes were transferred through

two washes of TL-Hepes to remove spermatozoa. Zygotes were subsequently transferred into Falcon 15 ml conical tubes (Scientific Products, McGraw Park, IL) and the cumulus cells removed by vortexing at high speed for 1 min. After vortexing, the remaining cumulus cells were denuded by manual pipetting and zygotes were mounted on glass slides with coverslips. Slides were placed in fixative (ethanol:acetic acid, 3:1) for at least 24 h and then stained with 1% orcein stain (Sigma Chemical Co., St Louis, MO). Slides were evaluated on an inverted microscope ($\times 40$) to assess normal fertilization (two pronuclei).

Protein preparation

Bovine oviducts from various stages of the oestrous cycle (Boice *et al.*, 1990a) were collected at an abattoir and placed in sterile saline on ice for transport to the laboratory. Excess connective tissue was removed and the oviducts were then minced and placed in an explant culture system for 24 h (Verhage *et al.*, 1988). The procedures used for partial purification of bOGP have been described in detail (Boice *et al.*, 1992; Schmidt *et al.*, 1997). Briefly, the proteins present in the culture medium were separated on a Sephacryl S-200 column (Pharmacia LKB, Piscataway, NJ). The proteins eluting in the void volume were dialysed against Millipore water (Milford, MA) using 50 000 molecular weight cut-off tubing (Spectra/Por, Spectrum, Houston, TX) and were then sterile filtered. Protein concentrations were measured using the method of Lowry *et al.* (1951). Partially purified bOGP was aliquoted, lyophilized and stored at -20°C .

Antibody production

The procedures for the production of a polyclonal antibody to bOGP were as described by Boice *et al.* (1990a). Oviductal tissue culture media proteins present in the void volume fraction of the S-200 column were separated by one-dimensional (1-D) SDS-PAGE on 5% acrylamide gels and were immediately transferred to nitrocellulose membranes (BA 85, Schleicher and Schuell, Keene, NH) at 200 mA constant current for 14–18 h using a Hoefer Trans-Blot system (Hoefer Scientific, San Francisco, CA) according to the procedure of Towbin *et al.* (1979). The nitrocellulose membranes were stained with Ponceau S (Sigma Chemical Co., St Louis, MO) to visualize proteins and the upper band of a doublet at 97 kDa was cut out and dissolved in dimethyl sulfoxide (DMSO). Approximately 100 µg of protein was dissolved in 500 µl DMSO and an equal volume of Freund's complete adjuvant. A male rabbit was bled from the ear vein and then injected with the protein emulsion at multiple intradermal sites along the back. Booster injections were given at 2, 4, 6, 7 and 8 weeks after the initial injection. Booster injections were approximately 100 µg of protein dissolved in DMSO emulsified in Freund's incomplete adjuvant. The antibody used in this study was from blood drawn 10 and 12 weeks after the initial injection. Blood from these collections was pooled and the IgG fraction was purified by ammonium sulphate precipitation followed by ion-exchange chromatography on diethylamino ethyl cellulose (DEAE cellulose; Sigma Chemical Co., St Louis, MO). The IgG

fraction was further purified by affinity chromatography on bovine serum-agarose (Sigma Chemical Co., St Louis, MO) to remove any antibodies to serum proteins. The isolated IgG fraction (anti-bOGP IgG) was dialysed against water and sterile filtered. Total protein concentration was measured by the procedure of Lowry *et al.* (1951), and aliquots were then lyophilized and stored at -20°C .

Western blot analysis

Proteins (approximately 25 μg) present in the void volume fraction of the S-200 column were separated by 1-D SDS PAGE on 7.5% acrylamide gels and were immediately transferred to nitrocellulose membranes as described previously. Anti-bOGP IgG was used as the primary antibody to detect bOGP in the void volume sample of bovine oviductal tissue culture media. Peroxidase conjugated goat anti-rabbit IgG (BioRad, Hercules, CA) was used as the second antibody and binding was detected using the BioRad peroxidase anti-peroxidase kits according to the manufacturer's instructions.

Experiment 1

Experiment 1 examined the effects on fertilization rates of inclusion of bOGP in the *in vitro* insemination period. Preliminary experiments in this study indicated that 100 μg bOGP ml^{-1} was optimal for effects on fertilization and this concentration was used in all experiments. The effects of protein supplement (albumin or bOGP) and final sperm concentration (0.5, 0.25 or 0.125×10^6 spermatozoa ml^{-1}) on fertilization rates were examined by a 2×3 factorial design. The numbers of oocytes allocated to individual treatment groups were as follows: 0.5×10^6 spermatozoa ml^{-1} , 112 and 113 oocytes for albumin and bOGP, respectively; 0.25×10^6 spermatozoa ml^{-1} , 116 and 126 oocytes for albumin and bOGP, respectively; and 0.125×10^6 spermatozoa ml^{-1} , 125 and 124 oocytes for albumin and bOGP, respectively. A final sperm concentration of 0.5×10^6 spermatozoa ml^{-1} is used for routine IVF procedures in this laboratory (Thibodeaux *et al.*, 1995). Fertilization dishes were allowed to equilibrate for 1 h at 39°C and 5% CO_2 before introduction of oocytes and spermatozoa. Oocytes were placed in the fertilization drops containing protein treatments and the 50 μl aliquot of sperm suspension was then added. There was no preincubation of oocytes or spermatozoa with the protein before initiating the experiment.

Experiment 2

Experiment 2 was conducted to determine whether the enhanced fertilization rates observed in Expt 1 were due to the biological activity of bOGP. Oocytes were randomly assigned to one of the following treatment groups: 100 μg albumin ml^{-1} (170 oocytes), 100 μg bOGP ml^{-1} (187 oocytes), 400 μg anti-bOGP IgG ml^{-1} (209 oocytes) or 100 μg bOGP and 400 μg anti-bOGP IgG ml^{-1} (186 oocytes). Preliminary experiments in this study indicated that 400 μg anti-bOGP IgG ml^{-1} was required to inhibit the effects of 100 μg bOGP

Table 1. Experimental design for supplementation of media with bovine oviductal glycoprotein or albumin at different phases in bovine IVF

During 2 h preincubation		During 16–18 h insemination	Number of oocytes
Oocytes	Spermatozoa		
Albumin	Albumin	Albumin	150
bOGP	Albumin	Albumin	134
Albumin	bOGP	Albumin	106
Albumin	Albumin	bOGP	158
bOGP	bOGP	Albumin	176
bOGP	Albumin	bOGP	111

bOGP: bovine oviductal glycoprotein.

ml^{-1} . Based on the results of Expt 1, the final sperm concentration used was 0.125×10^6 spermatozoa ml^{-1} and all treatment groups were preincubated for 1 h in the fertilization drops before introducing oocytes and spermatozoa.

Experiment 3

Experiment 3 examined whether the enhanced fertilization rates observed in Expts 1 and 2 were due to an effect on the oocyte, spermatozoon or both during the insemination process. Oocytes and spermatozoa were exposed to albumin or bOGP during different phases of the IVF process. After the maturation period, oocytes were washed in fertilization medium and randomly assigned to various preincubation treatment groups (Table 1). After the 2 h preincubation period (39°C and 5% CO_2), oocytes and spermatozoa were washed in the designated albumin or bOGP treatments to be applied during the 16–18 h insemination period (39°C and 5% CO_2). The insemination medium was supplemented with the albumin and bOGP treatments at a concentration of 100 μg ml^{-1} and the final sperm concentration was 0.125×10^6 spermatozoa ml^{-1} .

Experiment 4

Experiment 4 was conducted to determine whether the enhanced fertilization rates observed after exposure of oocytes to bOGP in Expt 3 were due to the biological activity of bOGP. Oocytes were randomly assigned to one of the following treatment groups: 100 μg albumin ml^{-1} (100 oocytes), 100 μg bOGP ml^{-1} (90 oocytes) or 100 μg bOGP and 400 μg anti-bOGP IgG ml^{-1} (90 oocytes). Oocytes were preincubated for 2 h (39°C and 5% CO_2), washed in fertilization medium and placed in fertilization drops (containing albumin only). The final sperm concentration was 0.1×10^6 spermatozoa ml^{-1} .

Statistical analysis

In all experiments each replicate (experimental day consisting of a batch of oocytes) oocytes were randomly assigned across all treatments. All experiments were replicated three times. Data from Expt 1 were analysed by two-way least

Fig. 1. Serum and partially purified bovine oviductal glycoprotein (bOGP) separated on one-dimensional SDS-PAGE (25 μ g protein per lane). Lane a is stained with Coomassie blue; lanes b and c are immunoblots using the polyclonal antibody anti-bOGP IgG. Lanes a and c, partially purified bOGP; lane b, bovine serum. Note the major doublet band at approximately 97 kDa in lanes a and c (arrowhead) and the absence of crossreacting antigens in lane b.

squares analysis of variance (ANOVA) with SigmaStat (Jandel Scientific, San Rafael, CA) statistical analysis software. Data from Expts 2, 3 and 4 were analysed by one-way least squares ANOVA. In all experiments, least-square means were compared using Tukey's highest significant difference test, and a probability of $P < 0.05$ was considered statistically significant.

Results

Western blot

Approximately 25 μ g of bovine serum protein and partially purified (35–50% based on SDS-PAGE analysis; Fig. 1, lane a) S-200 column void volume fluid from bovine oviductal culture media were analysed by western blotting. Anti-bOGP IgG was used at a concentration of 1.35 μ g ml⁻¹. A doublet band of crossreacting antigens (Fig. 1) was observed at approximately 97 kDa in the bovine oviduct sample (Fig. 1, lane c), whereas crossreacting antigens were absent in bovine serum (Fig. 1, lane b). These results are similar to the observations of other studies using different polyclonal antibodies to bOGP (Boice *et al.*, 1990a; Abe *et al.*, 1993; Gandolfi *et al.*, 1993).

Experiment 1

The effects of bOGP on fertilization rates were evaluated by reducing the concentration of spermatozoa used during the IVF

Fig. 2. Effect of bovine oviductal glycoprotein (bOGP) (100 μ g ml⁻¹) and sperm concentration on fertilization rates of bovine oocytes *in vitro*. (○) bOGP; (●) albumin. Values are least square mean ($\pm$ SEM) fertilization rates derived from three replicates. Different letters above or below the error bars indicate values are significantly different ($P < 0.05$).

process (Fig. 2). Within the albumin treatment, there was a significant decrease ($P < 0.05$) in fertilization rate with decreasing sperm concentration. Within the bOGP treatment, there was no difference in fertilization rate between the 0.5 and 0.25×10^6 spermatozoa ml⁻¹ and the 0.25 and 0.125×10^6 spermatozoa ml⁻¹ concentrations. However, a decrease ($P < 0.05$) in fertilization rate was observed between the 0.5 and 0.125×10^6 spermatozoa ml⁻¹ concentrations. The albumin treatment had a significantly lower ($P < 0.01$) fertilization rate at 0.125×10^6 spermatozoa ml⁻¹ compared with the bOGP treatment.

Experiment 2

Experiment 1 demonstrated that incubation of oocytes and spermatozoa with bOGP enhanced fertilization rate at a low sperm concentration (0.125×10^6 spermatozoa ml⁻¹). In Expt 2 the effects of bOGP (100 μ g ml⁻¹) on fertilization rates were blocked by the inclusion of anti-bOGP IgG (Fig. 3). Oocytes and spermatozoa incubated with bOGP had a significantly higher ($P < 0.05$) fertilization rate compared with all other treatment groups. There were no differences in fertilization rates between the albumin, anti-bOGP IgG, and bOGP and anti-bOGP IgG treatment groups. Therefore, the biological activity of bOGP was completely inhibited by the addition of anti-bOGP IgG during *in vitro* insemination.

Experiment 3

Preincubation of oocytes with 100 μ g bOGP ml⁻¹ for 2 h before *in vitro* insemination significantly ($P < 0.05$) increased fertilization rates compared with the control group, whereas

Fig. 3. Effect of bovine oviductal glycoprotein (bOGP) and anti-bOGP IgG on fertilization rates of bovine oocytes *in vitro* using a final sperm concentration of 0.125×10^6 spermatozoa ml^{-1} . The concentration of protein was $100 \mu\text{g ml}^{-1}$ for the albumin and bOGP treatments and $400 \mu\text{g ml}^{-1}$ for the anti-bOGP IgG treatment. The bars indicate the least square mean ($\pm$ SEM) fertilization rates derived from three replicates. * indicates a significant difference ($P < 0.05$) compared with all other treatment groups.

Fig. 4. Effect of bovine oviductal glycoprotein (bOGP) or albumin (both at $100 \mu\text{g ml}^{-1}$) on fertilization rates during different phases of the bovine IVF process using a final sperm concentration of $0.125 \times 10^6 \text{ ml}^{-1}$. Oocytes and spermatozoa were preincubated for 2 h before insemination with albumin or bOGP, rinsed, and introduced into albumin or bOGP insemination treatments for 16–18 h. The bars indicate the least square mean ($\pm$ SEM) fertilization rates derived from three replicates. Different letters above the error bars indicate values are significantly different ($P < 0.05$).

preincubation of spermatozoa with bOGP was the same ($P > 0.05$) as observed in the control group (Fig. 4). The presence of bOGP during the 16–18 h insemination period also significantly ($P < 0.05$) increased fertilization rates compared with the control group, as also observed in Expts 1 and 2 and in the oocyte preincubation group in Expt 3. Preincubating

Fig. 5. Effect of bovine oviductal glycoprotein (bOGP) ($100 \mu\text{g ml}^{-1}$) and anti-bOGP IgG ($400 \mu\text{g ml}^{-1}$) on fertilization rates of bovine oocytes preincubated for 2 h before IVF using a final sperm concentration of $0.1 \times 10^6 \text{ ml}^{-1}$. Oocytes were washed in albumin and standard IVF was conducted for 16–18 h in albumin. The bars indicate the least square mean ($\pm$ SEM) fertilization rates derived from three replicates. * indicates a significant difference ($P < 0.05$) compared with all other treatment groups.

both spermatozoa and oocytes with bOGP also resulted in a significant ($P < 0.05$) increase in fertilization rates, although this was not greater than that observed for preincubation of oocytes. Including bOGP in the oocyte preincubation media and during the 16–18 h insemination period resulted in a significant ($P < 0.05$) increase in fertilization rate, which was the same as that observed when oocytes were preincubated with bOGP.

Experiment 4

Experiment 3 demonstrated that preincubation of oocytes for 2 h with bOGP significantly ($P < 0.05$) enhanced fertilization rates compared with the albumin control. In Expt 4 the inclusion of a polyclonal antibody to bOGP (anti-bOGP IgG) was used to investigate whether the effect of bOGP on oocytes was due to the biological activity of the glycoprotein. Oocytes preincubated with bOGP had a higher ($P < 0.05$) fertilization rate compared with all other treatment groups (Fig. 5). The effects of bOGP were blocked by the inclusion of anti-bOGP IgG during the preincubation step.

Discussion

IVF techniques are well established and the fertilization rates achieved with well defined protocols are 80–90%. However, oocytes are exposed to very high concentrations of spermatozoa during IVF which may ultimately have a detrimental effect

on embryonic development and implantation since dying spermatozoa produce oxygen radicals (Aitken, 1995; Aitken *et al.*, 1996). The oviduct probably provides the optimal conditions for early reproductive events, and the ability to mimic this environment may enhance fertilization and early embryonic development and implantation since few spermatozoa are present in the ampulla at the time of fertilization (Hunter, 1988). An important constituent of the oviductal environment is a high molecular weight OGP. This secretory protein is well characterized in several species (for a review see Verhage *et al.*, 1997) including cattle (Boice *et al.*, 1990a, 1992; Gerena and Killian, 1990; Abe *et al.*, 1995).

The results of the present study demonstrated that inclusion of a partially purified bOGP preparation during the 16–18 h insemination period enhanced fertilization rates compared with albumin controls when sperm concentrations were reduced from 0.5 to 0.125×10^6 spermatozoa ml^{-1} . This effect is probably due to bOGP since preincubating bOGP with anti-bOGP IgG blocked the effect, whereas anti-bOGP IgG had no effect on fertilization rates. These data support previous reports in other species in which incubation of both gametes with OGP increased sperm binding to ovarian oocytes and sperm penetration rates. Schmidt *et al.* (1997) reported that the incubation of hamster ovarian oocytes and spermatozoa with hamster OGP in a sperm binding assay resulted in a twofold increase in sperm binding compared with controls. O'Day-Bowman *et al.* (1996) reported that the co-incubation of human spermatozoa and human zona pellucida with human OGP in a hemizona assay resulted in a threefold increase in sperm binding compared with controls. In both studies the effect on sperm binding was blocked by specific antibodies generated against human (O'Day-Bowman *et al.*, 1996) and hamster OGP (Schmidt *et al.*, 1997). Boatman and Magnoni (1995) reported that capacitated hamster spermatozoa incubated with ovarian oocytes (not exposed to endogenous OGP) had threefold higher penetration rates in the presence of hamster OGP compared with the controls. Therefore, OGP facilitates sperm binding to the zona pellucida, sperm penetration of the zona pellucida, and fertilization. In addition, including bOGP in IVF protocols may allow sperm concentrations to be significantly reduced while maintaining acceptable fertilization rates, and improve both embryonic development and implantation rates.

Oocytes and spermatozoa were co-incubated with bOGP during the fertilization period in the Expts 1 and 2. Therefore, the improved fertilization rates observed in these experiments could be due to the effect of bOGP on one of the gametes or a synergistic effect on both gametes. Before fertilization *in vivo* both gametes are exposed to OGP. Several studies have shown that OGP binds to the zona pellucida and perivitelline space of oviductal oocytes (Boice *et al.*, 1990a, 1992; Wegner and Killian, 1991; Kan and Roux, 1995). Binding of OGP to oocytes occurs very rapidly once the oocyte enters the oviduct and the glycoprotein remains associated with the zona pellucida even after vigorous washing (Reuter *et al.*, 1994). OGP has also been reported to associate with bovine (King and Killian, 1994) and hamster (Boatman and Magnoni, 1995) spermatozoa during incubation *in vitro*. However, in similar studies *in vitro*, OGP did not associate with human (Reuter *et al.*, 1994) or sheep (Hill and Nancarrow, 1993) spermatozoa. In addition, it

is not known whether OGP binds to spermatozoa in any species *in vivo*.

Preincubation of oocytes with bOGP resulted in a significant increase in fertilization rates which was similar to that observed when bOGP was included in the 16–18 h fertilization period (oocytes and spermatozoa exposed). However, when spermatozoa were preincubated with bOGP no increase in fertilization rate was observed, nor was there a synergistic effect when both gametes were preincubated with bOGP. The significant increase in fertilization rates observed when oocytes were preincubated with bOGP is likely due to the biological activity of bOGP, since inclusion of anti-bOGP IgG during culture blocked this effect. In addition, Staros and Killian (1997) incubated bovine oocytes for 2.5 h in bOGP purified from oviductal fluid and observed a significant increase in fertilization rates compared with oocytes incubated in BSA alone. These results suggest that the facilitatory effect of OGP on early reproductive events is primarily mediated through interaction of the protein with the oocyte, possibly through association with the zona pellucida and perivitelline space.

There was no effect on fertilization rates when spermatozoa were preincubated with bOGP. This finding is contradictory to previously published data. King *et al.* (1994) reported an increase in bovine sperm capacitation during 4 h incubation in the presence of bOGP compared with controls. The bOGP used in this study was purified from oviductal fluid and used at a concentration of approximately 1 mg ml^{-1} . In addition, spermatozoa capacitated in the presence of bOGP significantly increased fertilization rates compared with spermatozoa capacitated in the absence of bOGP (King *et al.*, 1994). The effect of bOGP was blocked when an antibody generated against bOGP was included in the capacitation media. The discrepancy between the present results and those of King *et al.* (1994) may be due to different experimental designs. King *et al.* (1994) used bOGP purified from oviductal fluid and exposed spermatozoa to bOGP during capacitation, whereas in the present study, partially purified bOGP released during culture was used after capacitation. In addition, different bOGP concentrations ($< 100 \mu\text{g ml}^{-1}$ versus approximately 1 mg ml^{-1}) and sperm concentrations ($0.125 \times 10^6 \text{ ml}^{-1}$ versus $0.5 \times 10^6 \text{ ml}^{-1}$) were used. It has also been reported that bOGP maintains sperm motility and viability (Abe *et al.*, 1995). Additional studies are needed in cattle and other species to determine the effect of OGP on sperm physiology. This may not be possible until highly purified samples of endogenous OGP or biologically active recombinant OGP are available.

In conclusion, this study shows that bOGP significantly enhances fertilization rates when sperm concentrations are reduced in current IVF protocols. The observed effect appears to be primarily mediated through the interaction of OGP with the oocyte. Preliminary studies suggest that this effect may also extend to embryonic development (Martus *et al.*, 1997). The role of OGP in fertilization and early embryonic development has important implications for current IVF protocols. Including bOGP in bovine IVF protocols may allow sperm concentrations to be significantly reduced while maintaining acceptable fertilization rates, and improve both embryonic development and implantation rates.

The research was supported in part by NIH Grant HD20571 (H. G. Verhage) and a grant from the Hillcrest Medical Center Foundation (J. K. Thibodeaux).

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